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Potent activity of a PK/PBAN analog with an (*E*)-alkene, *trans*-Pro mimic identifies the Pro orientation and core conformation during interaction with HevPBANR-C receptor

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ABSTRACT

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) family plays a multifunctional role in an array of important physiological processes in insects, including regulation of sex pheromone biosynthesis in moths. A cyclic PK/PBAN analog (*cyclo*[NTSFTPRL]) retains significant activity on the pheromonotropic HevPBANR receptor from the tobacco budworm *Heliothis virescens* expressed in CHO-K1 cells. Previous studies indicate that this rigid, cyclic analog adopts a type I β-turn with a *trans*Pro over residues TPRL within the core PK/PBAN region. An analog containing an (*E*)-alkene, *trans*-Pro mimetic motif was synthesized, and upon evaluation on the HevPBANR receptor found to have an EC_{50} value that is not statistically different from a parent C-terminal PK/PBAN hexapeptide sequence. The results, in aggregate, provide strong evidence for the orientation of Pro and the core conformation of PK/PBAN neuropeptides during interaction with the expressed PBAN receptor. The work further identifies a novel scaffold with which to design mimetic PBAN analogs as potential leads in the development of environmentally favorable pest management agents capable of disrupting PK/PBAN-regulated pheromone signaling systems.

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1. Introduction

The pyrokinin/pheromone biosynthesis activating neuropeptide (PK/PBAN) peptides represent a multifunctional family that plays a significant role in the physiology of insects. The first member of the family, leucopyrokinin (LPK), was discovered in the cockroach *Leucophaea maderae* in 1986[1] and since then over 30 peptides have been identified, all of which share the common C-terminal pentapeptide FXPRL-amide (X = S, T, G or V).[2,3] Functions of the PK/PBAN family include stimulation of sex pheromone biosynthesis in moths,[2–6] mediation of key functions associated with feeding (gut muscle contractions),[7,8] development (embryonic diapause, pupal diapause and pupariation),[9–14] and defense (melanin biosynthesis)[15,16] in a variety of insects. Studies indicate that all of the above functions can be stimulated by more than one PK/PBAN peptide (regardless of the identity of the variable X position in the C-terminal pentapeptide), and that the peptides do not exhibit species specificity.[2,16]

Sex pheromones provide important olfactory signals for species-specific courtship and mating. In some butterfly and moth species, synthesis and release of pheromones are regulated by pheromone biosynthesis activating neuropeptides (PBANs). The first PBANs identified from brain and subesophageal ganglia contain 33 or 34 amino acids, amidated at the C-terminus.[17–21] The active core of the PK/PBAN peptides for pheromonotropic activity has been identified as the conserved C-terminal pentapeptide.[7,22–24] A comparative study of the pheromonotropic activity of a series of natural PK/PBAN peptides in the moth *Helicoverpa zea* indicated that the most active features a Thr in the variable X position of the pentapeptide core; although the activity of the fragment-analogs FSPRLa and FTPRLa are nonetheless similar.[25] Despite significant advances over the last decades,[26,27] many questions regarding the precise functional roles of PBANs remain.

Recent efforts have focused on identification and tissue-specific expression of PBAN receptors. Analysis of the *Drosophila melanogaster* genome led to a prediction that 44 G protein-coupled

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receptors (GPCRs) mediate peptide hormone signaling.[28] It has been demonstrated previously that GPCRs CG8795 and CG8784 are highly sensitive to *D. melanogaster* pyrokinin-2 (DrmPK-2), a neuropeptide with high C-terminal sequence similarity to PBAN.[29,30] Indeed, moth orthologs of CG8795 have been identified as functional PBAN receptors in the moth *H. zea*[31] and *Bombyx mori*.[32]

Mating disruption by field application of synthetic pheromones has become a viable strategy for integrated pest management (IPM) of agriculturally important Lepidopteran pests. It therefore seems likely that chemicals interfering with PBAN signaling could become novel insect control agents of the future. The identification of key chemical and conformational requirements for the interaction of PBAN with expressed receptors would aid in the development of agrochemical reagents, and lead to the design of biostable analogs that can provide further insights into possible broader physiological roles for PBAN.

In a previous publication, we had described the cloning and functional expression of a PBAN receptor (HevPBANR-C) from *H. virescens*.[24] The expressed receptor was shown to be sensitive and selective for PBAN and its analogs. Through systematic alanine substitution, the receptor-active ligand core, along with critical information within, was also identified.[24]

In this study, we provide compelling evidence for the orientation adopted by the Pro residue within the PBAN active core region, and therefore the preferred core conformation, during interaction of PK/PBAN peptides with an expressed PBAN receptor from *H. virescens* (HevPBANR-C). In addition, the work identifies a novel scaffold with which to design potent, mimetic pseudopeptide and/or non-peptide PK/PBAN analogs.

2. Materials and methods

The PK/PBAN analogs *cyclo*[Asn^1]LPK and [2-8,Asn^9]LPK were synthesized as described previously.[33,34]

2.1. Synthesis of Fmoc-Ser(OTBDMS)Ψ[(*E*)-CH=C]Pro–OH

The protected motif Fmoc-Ser-Ψ[(*E*)-CH=C]Pro–OH was synthesized as previously described by Wang et al.[35,36] Fmoc–Ser-Ψ[(*E*)CH=C]Pro–OH (465 mg, 1.12 mmol) and imidazole (381 mg, 5.60 mmol) were dissolved in DMF (4.0 mL), and TBDMSCl (422 mg, 2.80 mmol) was added. The mixture was stirred for 16 h, and then NH_4Cl (20 mL) was added. The mixture was stirred for an additional 50 min, and then diluted with EtOAc (30 mL), washed with NH_4Cl (2 × 10 mL), dried with $MgSO_4$, and concentrated. Chromatography on silica gel with 0.1% acetic acid/30% EtOAc/hexanes gave 450 mg (76%) of Fmoc–SerΨ[(*E*)CH=C]Pro–OH as a colorless foam. Mp 62–63 °C. ^{1}H NMR (DMSO-d_6) δ 7.88 (d, J = 7.4, 2H), 7.68 (d, J = 7.4, 2H), 7.41 (t, J = 7.5, 2H), 7.31 (t, J = 7.2, 2H), 7.28 (d, J = 8.5, 1H), 5.37 (d, J = 7.6, 1H), 4.27 (m, 2H), 4.16 (m, 2H), 3.50 (dd, J = 10.1, 6.7, 1H), 3.40 (dd, J = 9.9, 6.7, 1H), 3.17 (t, J = 7.1, 1H), 2.35 (m, 1H), 2.26 (m, 1H), 1.80 (m, 3H), 1.53 (m, 1H), 0.82 (s, 9H), -0.01 (d, J = 2.8, 6H). ^{13}C NMR (DMSO-d_6) δ 175.2, 156.2, 144.6, 144.5, 141.3, 128.1, 127.6, 125.8, 121.3, 120.7, 65.9, 65.3, 53.1, 49.6, 47.3, 30.1, 29.7, 26.3, 25.0, 18.5, -4.8, -4.9. Anal. Calcd for $C_{30}H_{39}NO_5Si$: C, 69.06; H, 7.53; N, 2.68. Found: C, 68.98; H, 7.62; N, 2.70.

2.2. Pseudopeptide synthesis

The peptidomimetic analog, Ac-Tyr-Phe-SerΨ [(*E*)-*CH*=*C*]Pro-Arg-Leu-NH_2 (PK-Etz) was synthesized manually by the solid-phase method, using the Fmoc-strategy and starting from 0.1 mM Rink amide resin (Novabiochem, 0.47 mM/g). The Fmoc protecting group was removed by 20% piperidine in DMF and the resin and later on the growing peptide-resin was washed with DMF, the MeOH and DCM. A fivefold excess of the respective Fmoc-amino acids was activated *in situ* using HBTU (0.9 equiv)/HOBt (1 equiv) in NMP and coupling reactions were base catalyzed with collidine. Amino acid side-chain protecting groups were TBDMS for Tyr and Ser(OTBDMS)Ψ[(*E*)-*CH*=*C*]Pro and Pbf for Arg. he synthesis of enantiomerically pure Fmoc-Ser(OTBDMS)Ψ[(*E*)-*CH*=*C*]Pro–OH is described above. The coupling of this Ser-*trans*-Pro isostere was mediated by HATU/HOAt instead of HBTU/HOBt. The completeness of each oupling reaction during synthesis was monitored by the Kaiser test. A second coupling was performed when the test was found positive. Cleavage of the peptide from the resin with side-chain deprotection was performed by treatment with TFA:H_2O:TIS (95.5:2.5:2.5 v/v/v, 10 ml/g peptide-resin) for 1.5 h. The cleaved peptide was precipitated with 20 volumes of diethyl ether, filtered, washed successively with more ether and air-dried. The resulting crude peptide was extracted with water and lyophilized.

The peptidomimetic analog was purified on a Waters C_{18} Sep Pak cartridge, and a Delta-Pak C_{18} reverse-phase column (8 × 100 mm, 15 μm particle size, 100 Å pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: Initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 ml/min. Delta-Pak C-18 retention time: t_R = 11.3 min. Amino acid analysis was carried out under previously reported conditions[37] and used to quantify the peptides and to confirm identity, leading to the following analysis: F[1.0], L[0.9], R[0.8], Y[1.0];. The identities of the peptide analogs were confirmed via MALDI-MS on a Kratos Kompact Probe MALDI-MS machine (Kratos Analytical, Ltd, Manchester, UK) with the presence of the molecular ion 806.4 [MH$^+$].

2.3. CHO cell expression

HevPBANR were expressed and assayed in wild type CHO-K1 cell lines. CHO-K1 cells were transfected with two plasmids containing ORFs of HevPBANR-C and codon-optimized aequorin as described.[24] Procedures used for CHO cell transfection and the receptor assay were described previously.[38]

3. Results

The activity of the conformationally restricted, cyclic pyrokinin/PBAN analog *cyclo*[Asn^1]LPK and its linear, N-terminally appended counterpart [2-8,Asn^9]LPK were compared with the natural 33-membered HezPBAN at concentrations of 1 and 100 nM on HevPBANR expressed in CHO-K1 cells. The N-terminally appended analog ([2-8,Asn^9]LPK) proved inactive at these two doses, indicating that appending the Asn to the N-terminus of a pyrokinin sequence is highly deleterious to interaction with the expressed receptor (Fig. 1A). Nonetheless, cyclization of [2-8,Asn^9]LPK, which led to *cyclo*[Asn^1]LPK, restored a large portion of the activity that was lost. While *cyclo*[Asn^1]LPK is not active at 1 nM, it exceeds a 70% maximal response at the 100 nM dose (Fig. 1A). Previous solution conformation studies indicate that *cyclo*[Asn^1]LPK adopts a rigid type I β-turn with a *trans*Pro orientation[33,34] (Fig. 2). Full dose-response curves were generated for analog PK-Etz (Ac-Tyr-Phe-SerΨ[(*E*)-*CH*=*C*]Pro-Arg-Leu-NH_2), containing a *trans*-Pro mimetic motif, along with a parent PK/PBAN hexapeptide sequence YFTPR-La. The EC_{50} values obtained for interaction with HevPBANR were 0.18 ± 0.067 nM for PK-Etz and 0.10 ± 0.046 nM for YFTPRLa, a dif-

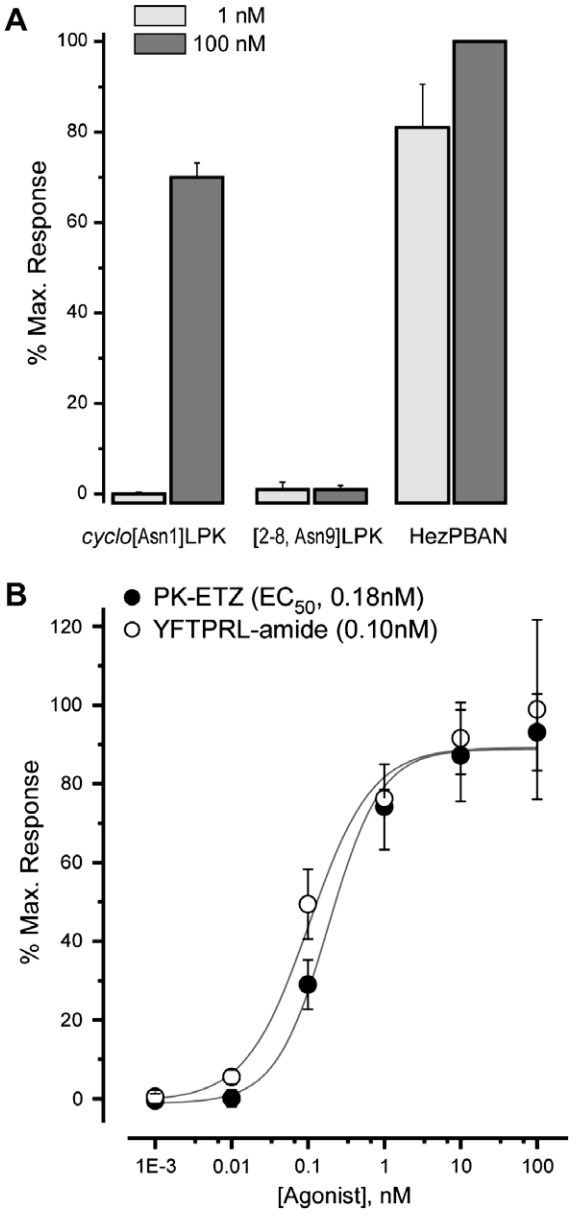

Figure 1. (A) Agonist activities of two related LPK derivatives (*cyclo*[Asn^1]LPK and the linear analog [2–8, Asn^9]LPK) on HevPBANR-C. The former is a cyclic version of the latter, which is [2–8]LPK with an Asn residue appended to the C-terminus. Luminescence response induced by 100 nM HezPBAN was set as 100%. (B) Luminescence responses of CHO cells co-expressing HevPBANR-C and aequorin for the analog PK-Etz and the PK/PBAN hexapeptide YFTPRLa are plotted as a function of various agonist concentrations. Each point is a mean value ± S.D. for percent of maximum response. Number in parentheses indicates EC_{50} value. The difference between the two EC_{50} values is not statistically significant.

ference which is not statistically significant (Fig. 1B). Therefore, PK-Etz is essentially equipotent with a parent PK/PBAN hexapeptide sequence on HevPBANR. It should be noted that the C-terminal pentapeptide core fragment-analogs in which the variable X position is occupied by either an S or a T show similar pheromonotropic activity in the heliothine moth *H. zea*.[25]

4. Discussion

The C-terminal pentapeptide FXPRLa is highly conserved and thus, shared by PBAN and other pyrokinins. It has further been identified as the ligand core for activity in pheromonotropic bioassays (X = S)[2,4,5,22] and the expressed PBAN receptor from the moth *H. virescens* (HevPBANR-C),[24] although the C-terminal hexapeptide YFXPRLa (X = S) exhibits much greater potency. In the pheromonotropic assay of the related heliothine insect *H. zea* indicates that the core PK/PBAN C-terminal pentapeptide sequence exhibits similar potency whether the variable X position is occupied by an S or a T.[25] Several turn conformations have been proposed for the core pentapeptide region based on NMR experiments of PBAN and/or core analogs in solution. Using the C-terminal hexapeptide PBAN analog [D-Phe,[29] 28–33]PBAN in an NMR solution conformation study, Wang et al. reported that it adopts a type II β-turn; although the authors concluded that this may have resulted from the conformational averaging of a type I β-turn and an extended structure.[39] Clark and Prestwich investigated the solution conformation of the natural HezPBAN and reported a type I' β-turn with a *cis*-Pro in the C-terminal pentapeptide region.[40] They found no interaction between the C-terminal turn and the rest of the PBAN peptide chain, providing evidence that the turn is the critical conformation recognized by the PBAN receptor. Drawbacks to the studies conducted by Wang et al. and Clark and Prestwich are that they were investigating highly flexible structures and NMR experiments were conducted in solutions containing organic solvents (Wang: DMSO; Clark/Prestwich: 2,2,2-trifluoroethanol), which can promote formation of secondary structure that is not necessarily relevant to the conformation adopted during receptor docking. Nachman et al. conducted a conformational study of the rigid, cyclic PK/PBAN analog *cyclo*[NTSFTPRL] (*cyclo*[Asn^1]LPK) in aqueous solution containing no organic solvents using a combination of NMR spectroscopic and molecular dynamics.[33,34] The specific conformation of this constrained, cyclic analog in aqueous solution was shown to be extremely rigid, featuring a *trans*-oriented Pro in the second position of a type-I β-turn over residues Thr-Pro-Arg-Leu within the core region. Indeed, a *trans*Pro is a defining characteristic of a type I β-turn.[41] The very large (for Thr-2, Thr-5, and Leu-8) and very small (for Ser-3 and Arg-7) coupling constants found indicated that the backbone of *cyclo*[Asn^1]LPK was rigidly held in a single or a few closely related conformations, since conformational averaging would have given averaged, intermediate values.[33] A comparison of these three solution conformation studies on PBAN analogs is outlined in Figure 2. Recently, a structure for the HezPBAN receptor has been predicted using the X-ray diffraction structure of the GPCR rhodopsin as a template; and this calculated structure has

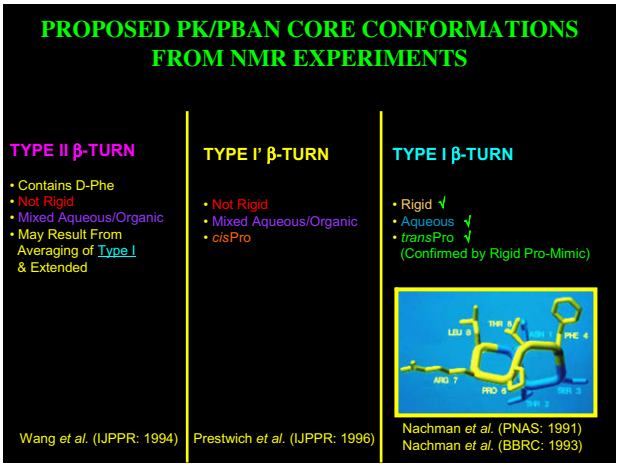

Figure 2. A comparison of proposed β-turn conformations of the PK/PBAN C-terminal pentapeptide core ligand based on three NMR solution conformation studies of PK/PBAN analogs. The computer graphics in the lower right corner illustrates the *trans*Pro, type I β-turn of the rigid, cyclic PK/PBAN analog *cyclo*[Asn1]LPK. This illustration was reproduced from Figure 3 of Ref. 34 with permission from Academic Press.

been used to build a binding model for the HezPBAN C-terminal hexapeptide fragment adopting each of the three proposed β-turn types. The model clearly supports the presence of a β-turn in the receptor bound conformation of PBAN core, but is not concise enough to provide evidence for the specific type of β-turn.[42]

Despite the conformational constraint imposed upon the cyclic PK/PBAN analog *cyclo*[Asn^1]LPK, it was found to retain 10% of the pheromonotropic activity of the 33-residue Bom-PBAN-I in a pheromonotropic bioassay in the silkworm *B. mori*,[34] the same percentage of activity retained by the linear C-terminal PBAN hexapeptide.

In this study, the conformationally constrained analog *cyclo*[Asn^1]LPK was evaluated on the expressed HevPBANR-C receptor from the moth *H. virescens* and found to retain a significant portion of the activity of the C-terminal hexapeptide pyrokinin/PBAN analog YFTPRLa. Although it appeared to be less potent, at 100 nM the cyclic analog elicited a 70% maximal response as compared with 91% and 100% for YFTPRLa and HezPBAN, respectively. The process of cyclization necessarily appends a residue to the C-terminus that is not present in the natural peptides. Thus, it is also valid to compare the activity of the cyclic analog to a linear analog that features an Asn residue appended to the N-terminus, as in TSFTPRLNa. On the expressed HevPBANR-C receptor, this linear analog demonstrates no activity at either 1 or 100 nM concentrations. It is clear that appending a residue to the C-terminus of the pyrokinin/PBAN sequence is deleterious to activity, though the cyclic analog *cyclo*[Asn^1]LPK nevertheless retains significant activity on the HevPBANR-C receptor and in pheromonotropic bioassays. The conformation induced by the rigidly constrained analog *cyclo*[Asn^1]LPK apparently allows for restoration of some of the activity lost in its linear, appended counterpart.

In order to provide more definitive evidence that a *trans*Pro, and a type I β-turn, represented the active conformation for pheromonotropic activity, the PK/PBAN analog PK-Etz, incorporating a *trans*-Pro isostere, was evaluated in the HevPBANR-C receptor assay in this study. In PK-Etz, the peptide bond of the Pro is replaced with a rigid double bond that locks in the *trans* orientation[35] (Fig. 3). Analog PK-Etz demonstrated activity (EC_{50} = 0.18 ± 0.067 nM) on the HevPBANR receptor that was not significantly different than its C-terminal hexapeptide PK/PBAN analog equivalent YFTPRLa (EC_{50} = 0.10 ± 0.046 nM). The relatively potent agonist activity of PK-Etz provides strong evidence that a *trans*Pro represents an important conformational aspect of the interaction of PBAN with its receptor in the moth *H. virescens*. What impact does the establishment of a *trans*Pro orientation have on the preferred β-turn conformation? Previous NMR studies have led to the three proposed β-turn types (type I, type II and type I') for the PBAN core region discussed above (Fig. 2). Of the three studies, only Nachman et al.[33,34] used both a conformationally rigid PK/PBAN analog along with aqueous solutions free of added organic solvents that artificially promote the formation of secondary structure. Of further interest is the admission by Wang et al.[39] that their finding of a type II β-turn could have resulted from the conformational averaging of a type I β-turn (identified in the study by Nachman et al.[33,34]) and an extended conformation in the flexible analog used. The type I β-turn proposed by Nachman et al.[33,34] features a *trans*Pro that was clearly evident in the rigid, cyclic analog *cyclo*[Asn^1]LPK and has now been confirmed by the potent activity of PK-Etz, which locks in a *trans* orientation with an alkene bond that is unable to rotate. This finding is not consistent with the type I' β-turn proposed in the study by Clark and Prestwich[40] that used the highly flexible HezPBAN as it features a *cis*Pro and not a *trans*-Pro. The work described here not only provides evidence for the orientation of Pro and core conformation for the interaction of pyrokinin/PBAN peptides with the pheromonotropic receptor from *H. virescens*, but also identifies a scaffold with which to design mimetic PBAN analogs. Such analogs may provide leads in the development of novel insect-specific, environmentally favorable pest management agents capable of disrupting PK/PBAN-regulated pheromone signaling systems (Table 1).

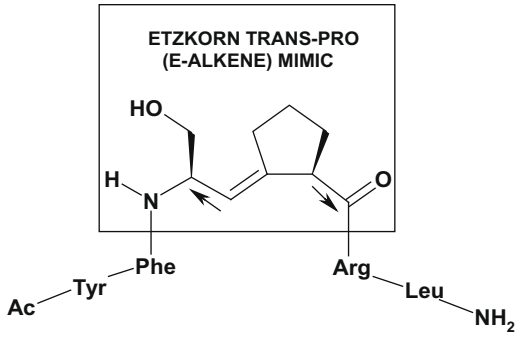

Figure 3. Structure of the analog PK-Etz (Ac-Tyr-Phe-SerΨ[*trans-CH*=*C*]Pro-Arg-Leu-NH_2), containing an (*E*)-alkene, *trans*Pro motif ('Etzkorn'). In this motif, the peptide bond that binds the amino group of the Pro is locked into a *trans* orientation by replacement with a double bond, which lacks the ability to rotate between *trans* and *cis* orientations as does a normal peptide bond.[35,36]

Table 1
Sequences of the compounds evaluated on the pheromonotropic HevPBANR-C receptor from *Heliothis virescens* expressed in CHO-K1 cells

Name	Sequence
HevPBAN	LSDDMPATPADQEMYRQDPEQIDSR–TKYFSPRLa
HezPBAN	LADDMPATPADQEMYRQDPEQIDSRRTKYFSPRLa
[28–33, Thr^{30}]PBAN	YFTPRLa
PK-Etz	Ac-YFSΨ[*trans*-CH=C]PRLa
cyclo[Asn^1]LPK	*Cyclo*[NTSFTPRL]
[2-8, Asn^9]LPK	SFTPRLNa

Ac: acetyl-; Etz: *trans*Pro Etzkorn mimetic component (see Fig. 3).

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